Enzymatic Esterification of Glycerol II. Lipase-Catalyzed Synthesis of Regioisomerically Pure 1(3)-*rac*-Monoacylglycerols

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Regioisomerically pure 1(3)-*rac*-monoacylglycerols are conveniently prepared in high yields (>75%) and in multigram quantities by enzymatic esterification of glycerol in the presence of various lipases *(Chromobacterium viscosum, Rhizopus delemar, Rhizomucor miehei)* with a variety of different acyl donors, such as free fatty acids, fatty acid alkyl esters, vinyl esters and triacylglycerols, as well as natural fats and oils. All reactions are carried out in aprotic organic solvents with low water content, namely *n*-hexane, diethyl ether, tBuOMe or mixtures of these solvents. Essential for the success of these transformations were the following two factors. First, the creation of an artificial interphase between the solvent-immiscible hydrophilic glycerol and the hydrophobic reaction medium by its adsorption onto a solid support. Second, a facile system for the separation of the desired monoacylglycerol from the reaction mixture, coupled with the continuous recycling of acyl donor and undesirable by-products.

KEY WORDS: Continuous product separation, direct esterification, irreversible transesterification, lipase, 1(3)-*rac*-monoacylglycerols, reversible transesterification, synthesis.

Regioisomerically pure 1(3)-*rac*-monoacylglycerols are potentially useful starting materials for numerous synthetic applications (1-3). They can be employed for the synthesis of defined triacylglycerols, phospholipids, glycolipids and the preparation of numerous enzyme agonists and antagonists. Conjugates of these molecules with various pharmaceuticals are potentially attractive as prodrugs or for their controlled release (4). Numerous applications of these odorless and colorless surfactants for cosmetic and pharmaceutical purposes are possible.

Mixtures of long-chain monoacylglycerols and other glycerides are widely employed as nonionic surfactants in the processing of foods and related applications (5,6). Isomeric mixtures of "monoglycerides" of this kind are normally produced by alcoholysis of the corresponding triacylglycerols with two equivalents of glycerol in the presence of metal catalysts at temperatures of 210–240°C (7,8). Only low yields of the desired compounds are usually obtained this way, the resulting products are frequently colored and not free of odor. "Monoglycerides" of higher purity (up to 90%) are obtained by molecular distillation of these crude mixtures. However, they still contain mixtures of regioisomers. Isomerically pure 1-(3)-monoacylglycerols are only obtained *via* classical synthetic routes based on suitable precursors like isopropylidene glycerol (9) or glycidol (10). It should be mentioned, however, that none of these products can be termed natural as defined by legislature.

Because lipases are well-known for their capability to hydrolyze and synthesize glycerides with marked regioselectivity, an enzymatic route to these target molecules seems to be highly attractive. Consequently, a number of research groups have been engaged in the synthesis of monoacylglycerols by lipase-catalyzed reactions (11–14). Recently it was reported that high yields of monoacylglycerols (up to 90%) were obtained by glycerolysis of a variety of natural fats and oils under carefully controlled conditions in the presence of various combinations of biocatalysts (15,16). Unfortunately, however, regioisomerically pure 1(3)-*sn*-monoacylglycerols cannot be obtained this way because the main products of lipase-catalyzed alcoholysis of triglycerides are 1,2-(2,3)-di- and 2-monoacylglycerols, respectively. Furthermore, under the protic (aqueous) reaction conditions employed, mixtures of regioisomers are always produced due to spontaneous acyl group migrations (13). Clearly, a facile method by which regioisomerically pure and possibly natural 1(3)-monoacylglycerols can be prepared in synthetically useful quantities would be highly desirable.

MATERIALS AND METHODS

All solvents were of technical grade and distilled before use. Lipase from *Rhizomucor miehei* (Lipozyme) was a gift from Novo Industri S/A (Bagsvaard, Denmark); lipases from *Rhizopus delemar* and *Pseudomonas* sp. were gifts from Amano Pharmaceutical Co. (Nagoya, Japan); and lipase from *Chromobacterium viscosum* was purchased from Toyo Jozo Co. Ltd. (Shizuoka, Japan). Regioselectivity of the lipases was determined as described previously (17). Other materials were purchased from Fluka Chemie AG (Buchs, Switzerland).

Product composition and purity was checked by gas-liquid chromatography (GLC) of the corresponding trimethylsilylethers (TMS-ethers) on a Carlo Erba (Milan, Italy) Fractovap 2150 capillary gas chromatograph equipped with a 25 m × 15 mm SE-30-fs column (Macherey-Nagel, Düren, Germany). Helium was used as the carrier gas with a pressure of 0.6 bar; cold split injection (1:25) was used. The detector temperature was 350°C, and the following temperature program was used: 200°C (1 min isothermal) to 320°C (3°C/min, 1 min isothermal).

Adsorption of glycerol onto silica gel: typical procedure. Equal amounts of water-free glycerol and silica gel (230–400 mesh) were mechanically mixed until the glycerol liquid was completely adsorbed and a free-flowing "dry" powder was obtained as described previously (18). The adsorbed glycerol can be stored under anhydrous conditions for several months.

*Synthesis of 1(3)-*rac*-monoacylglycerols via irreversible acyl transfer: typical procedure.* Glycerol (4.6 g, 50 mmol) adsorbed onto 4.6 g of silica gel (70–230 mesh) was suspended in 50 mL of tBuOMe. To the suspension was added 2.26 g (10 mmol) of vinyl laurate and 100 mg of lipase from *Rhizomucor miehei* (Lipozyme). The mixture was stirred at room temperature for 24 h, and the reaction progress was monitored by thin-layer chromatography (TLC). After removal of the solid components (immobilized biocatalyst and silica gel) by filtration and evaporation of the solvent, a crude reaction mixture was obtained that contained about 70% of 1(3)-monoacylglycerol with 1,3-diacylglycerol as major by-product (Table

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TABLE 1

Lipase-Catalyzed Synthesis of Regioisomerically Pure 1(3)-*rac*-Monoacylglycerols; Dependence of Yields on the Ratio of Glycerol and Acyl Donor

Product	Enzyme[a]	Acyl donor	Ratio (glycerol/acyl donor)	Time (h)	Conv. (%)[b]	MG content (crude)[c]	Yield[d] (%)	Purity (%)
1-Monovalerin	A	Valeric acid	1:1	96	93	29	23	98[e]
1-Monovalerin	A	Valeric acid	5:1	48	95	65	57	98[e]
1-Monocaprylin	A	Vinyl caprylate	1:1	48	>98	28	23	98[f]
1-Monocaprylin	A	Vinyl caprylate	5:1	48	>98	67	60	98[f]
1-Monolaurin	B	Vinyl laurate	1:1	48	>98	30	22	>99[f]
1-Monolaurin	B	Vinyl laurate	2:1	24	>98	45	40	>99[f]
1-Monolaurin	B	Vinyl laurate	5:1	24	>98	70	60	>99[f]
1-Monolaurin	B	Vinyl laurate	10:1	24	>98	65	57	>99[f]
1-Monoolein	C	Oleic acid	1:1	96	90	33	25	98[e]
1-Monoolein	C	Oleic acid	5:1	72	94	70	65	98[e]

[a]Enzyme A, lipase from *Rhizopus delemar*; B, lipase from *Rhizomucor miehei*; and C, lipase from *Chromobacterium viscosum*.
[b]Based on acyl donor thin-layer chromatography.
[c]Determined by gas-liquid chromatography.
[d]Isolated yield, after purification.
[e]After column chromatography on boric acid-impregnated silica gel; eluent diethyl ether/*n*-hexane (1:1).
[f]After recrystallization from dry methanol.

1). The crude product was recrystallized from a mixture of *n*-hexane and diethyl ether in a ratio of 1:1 to yield 1.4 g (50%) of regioisomerically pure (>98%) 1-monolaurin, colorless crystals (m.p. 63–63.5°C). The purity was determined by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy and GLC.

*Synthesis of 1(3)-*rac*-monoacylglycerols via reversible acyl transfer or direct esterification: typical procedure.* Glycerol (4.6 g, 50 mmol) adsorbed onto 4.6 g of silica gel (70–230 mesh) was suspended in 50 mL of tBuOMe. To the suspension was added 2.0 g (10 mmol) of lauric acid [alternatively 2.2 g (10 mmol) methyl laurate], 2 g of 3Å molecular sieve (alternatively 4Å) and 100 mg of lipase from *Rhizomucor miehei* (Lipozyme). The mixture was stirred at room temperature for 48 h, and the reaction progress was monitored by TLC. After removal of the solid components (immobilized biocatalyst and silica gel) by filtration and evaporation of the solvent, a crude reaction mixture was obtained that contained about 68% of 1(3)-monoacylglycerol together with diacylglycerols (see Table 1). This product was separated by liquid chromatography on a boric acid-impregnated silica gel column (eluent diethyl ether/*n*-hexane, 1:1) to yield 1.4 g (50%) of regioisomerically pure (>98%) 1-monolaurin, colorless crystals (m.p. 63–63.5°C). The purity was controlled by ^{1}H-NMR spectroscopy and GLC.

*Synthesis of solid 1(3)-*rac*-monoacylglycerols via reversible acyltransfer; isolation and recycling of reaction by-products: typical procedure.* A 250-mL three-necked flask maintained at 25°C and equipped with an efficient mechanical stirrer (the reactor) was connected *via* a filter disc and a membrane pump with another 250-mL round-necked flask (the separator), which was placed in a cooling bath maintained at 2°C. The separator was connected with the reactor in the same manner to provide a solvent cycle.

Glycerol (4.6 g, 0.05 mol) adsorbed onto 10 g of silica gel (230–400 mesh) was suspended in the reactor. Trilaurin (10.5 g, 0.017 mol) and 300 mg of lipase from *Pseudomonas fluorescens* were added to the mixture, which was stirred and circulated through the system for 48 h. During the course of the reaction, 12.2 g (90%) 1(3)-monolaurin precipitated in the separator as a colorless solid, which was isolated by filtration under reduced pressure. The isomeric purity of the "crude" product as determined by GLC was 98%.

With the described method, other solid monoacylglycerols were produced in an analogous manner by using a variety of acyl donors, such as free fatty acids, fatty acid esters, synthetic triacylglycerols, natural palm kernel oil and coconut oil (Table 2). If free fatty acids or fatty acid methyl esters were used as acyl donors, molecular sieves (3Å or 4Å) were added into the reactor.

Preparation of trimethylsilyl derivatives for GLC analysis: typical procedure. The anhydrous glycerides (1 mg) and 0.2 mL of a mixture of *bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (10:1) were combined and heated at 70°C for three hours. This mixture (0.2 μL) was analyzed by GLC. If samples from liquid reaction mixtures were analyzed, the solvent was evaporated under a stream of dry nitrogen prior to their conversion.

RESULTS AND DISCUSSION

As outlined above, lipase-catalyzed hydrolysis or syntheses of glycerides would be a highly attractive route to regioisomerically pure monoacylglycerols. Unfortunately, however, due to spontaneous acyl group migration all attempts of this kind, carried out under aqueous or protic reaction conditions, were of only limited success.

We recently found that monoacylglycerols are quite stable toward acyl group migration in aprotic organic solvents with low water content (<2%) (17,19). Based on this observation, the synthesis of monoacylglycerols by direct enzyme-catalyzed esterification of glycerol would provide a facile route to these target molecules. Unfortunately, however, hydrophilic glycerol is immiscible with nonpolar organic solvents, and all attempts for its enzymatic esterification in these media have been unsuccessful (20). This problem can be overcome easily by prior adsorption of glycerol onto a solid support (18). Presum-

TABLE 2

Lipase-Catalyzed Synthesis of 1(3)-*rac*-Monoacylglycerols;[a] Isolation of Monoacylglycerols and Recycling of By-Products

Product	Acyl donor	Solvent mixture	Time[b] (h)	Separator temp. (°C)	Yield[c] (%)	Purity[d] (%)
1-Monocaprin	Capric acid	Et_2O/hexane, 1:1	48	−2	90	98
1-Monoundecanoin	Undecanoic acid	Et_2O/hexane, 1:1	48	0	87	96
1-Monoundec-10-enoin	10-Undecenoic acid	Et_2O/hexane, 1:1	48	−2	85	97
1-Monolaurin	Vinyl laurate	Et_2O/hexane, 1:1	48	2	90	98
1-Monotridecanoin	Tridecanoic acid	Et_2O/hexane, 1:1	48	4	89	97
1-Monomyristin	Trimyristin	Et_2O/hexane, 3:2	72	4	90	95
1-Monopentadecanoin	Pentadecanoic acid	Et_2O/hexane, 3:2	48	8	91	96
1-Monopalmitin	Tripalmitin	Et_2O/hexane, 3:2	72	8	88	98
1-Monostearin	Tristearin	Et_2O/hexane, 3:2	72	10	88	98
1-Palm kernel monoglyceride	Palm kernel oil	Et_2O/hexane, 3:2	72	−2	87	97[e]
1-Coco monoglyceride	Coconut oil	Et_2O/hexane, 3:2	48	−2	90	96[f]

[a]Lipase from *Pseudomonas fluorescens*.
[b]Time required for 98% conversion of the acyl donor.
[c]Isolated yields.
[d]Without further purification.
[e]Mixture of 1-monoacylglycerols (m.p. 59–62°C, fatty acid composition 50% C-12, 35% C-14).
[f]Mixture of 1-monoacylglycerols (m.p. 60–63°C, fatty acid composition 55% C-12, 30% C-14).

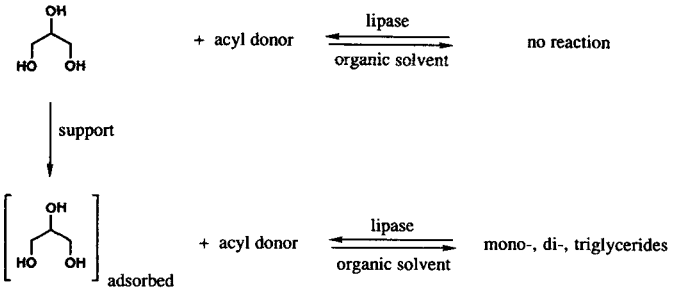


FIG. 1. Esterification of glycerol in organic solvents.

ably this process creates an artificial liquid-liquid interphase generally thought to be involved in lipase-catalyzed transformations of glycerides, *e.g.*, natural fats and oils. This reaction scheme is outlined in Figure 1.

Based on these initial results, we were able to develop a simple, one-step synthetic route to regioisomerically pure 1(3)-monoacylglycerols by direct esterification of glycerol with a variety of lipases and acyl donors including fatty acids, fatty acid alkyl and alkenyl esters, synthetic triacylglycerols and natural fats and oils. In typical experiments, glycerol and silica gel were first mixed mechanically until a free-flowing "dry" powder was obtained, which could be used for the following enzymatic esterifications. These were carried out in nearly anhydrous organic solvents (preferably tBuOMe) with a variety of acyl donors including free fatty acids (Eq. [2]), fatty acid methyl esters (Eq. [3]) and fatty acid vinyl esters (Eq. [1]) in the presence of different lipases. For this, the glycerol preparations were suspended in tBuOMe, and the corresponding acyl donor and a 1,3-selective lipase (17) were added. The obtained mixtures were stirred at room temperature while the esterification reactions were monitored by TLC. After completion of the esterifications, both the enzyme and the solid support were removed simultaneously by simple filtration, while the esterification products remained in solution. After evaporation of the solvent, mixtures consisting largely of 1(3)-monoacylglycerols and 1,3-*sn*-diacylglycerols, together with other impurities (low amounts of the corresponding regioisomers), were obtained (Table 3).

Unfortunately, the yields of desired 1(3)-monoacylglycerols did not exceed 30–40%. At first glance, and in view of the high regioselectivities of many lipases regarding the primary positions of glycerides, it seemed possible to increase the yield by employing an excess of glycerol. As documented in Table 1, this was indeed the case, and regioisomerically pure monoacylglycerols could now be prepared in considerably improved yields compared to previous attempts. Liquid compounds were isolated in isomerically pure form by chromatography on boric acid-coated silica gel, while solid compounds were recrystallized from a mixture of diethyl ether and *n*-hexane. Both were characterized by ^1H- and ^{13}C-NMR spectroscopy (21) and, alternatively, by TLC (22) and GLC (23).

However, even with a ten-fold excess of glycerol the content of monoacylglycerols in the reaction mixture did not exceed 70%. Obviously, the primarily produced 1(3)-monoacylglycerols are considerably better substrates for the lipase catalyst compared to glycerol itself, resulting in the production of 1,3-diacylglycerols as major by-products. Furthermore, while solid products can be purified quite easily by recrystallization, even in large quantities, the problem of purification remains for large quantities of liquid monoacylglycerols obtained from unsaturated and/or short-chain fatty acids. Another disadvantage of this process is the fact that no triacylglycerols can be employed as acyl donors because mixtures of the regioisomeric monoacylglycerols are produced under the employed conditions (Fig. 2). Clearly, a practical and useful method for the preparation of regioisomerically pure 1(3)-monoacylglycerols had to fulfill the following requirements: preparation of synthetically useful quantities in high yields, no need for tedious purification steps; the use of triacylglycerols and natural fats and oils as acyl donors; and the potential for a continuing process.

As mentioned previously (Table 1), the esterification of glycerol with a variety of acyl donors produced mixtures of mono- and diacylglycerols, even if a large excess of glycerol is employed, requiring additional separation and

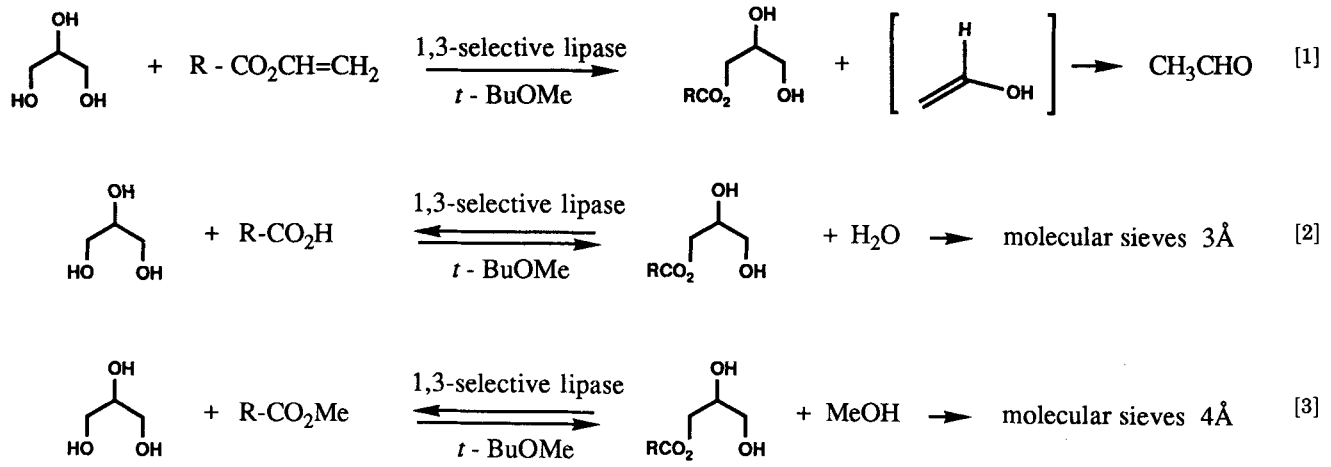


TABLE 3

Lipase-Catalyzed Synthesis of 1(3)-*rac*-Monoacylglycerols[a]

Product	Acyl donor[b]	Time (h)	Conv.[c] (%)	Composition of crude reaction mixture (%)[d] 1-MG	2-MG	DG	Yield[e] (%)	Purity[f] (%)
1-Monolaurin	Vinyl laurate	24	>98	30	3	67	25	>99
1-Monomyristin	Vinyl myristate	24	>98	32	3	65	27	>99
1-Monopentadecanoin	Pentadecanoic acid	48	94	40	4	56	31	>99
1-Monopalmitin	Vinyl palmitate	24	>98	35	3	62	30	>99
1-Monostearin	Vinyl stearate	24	>98	31	4	65	26	>99

[a]Lipase from *Rhizomucor miehei*.
[b]Stoichiometric ratio of glycerol and acyl donor.
[c]Based on acyl donor thin-layer chromatography.
[d]Determined by gas-liquid chromatography (GLC).
[e]Isolated yield, after purification.
[f]Determined by GLC.

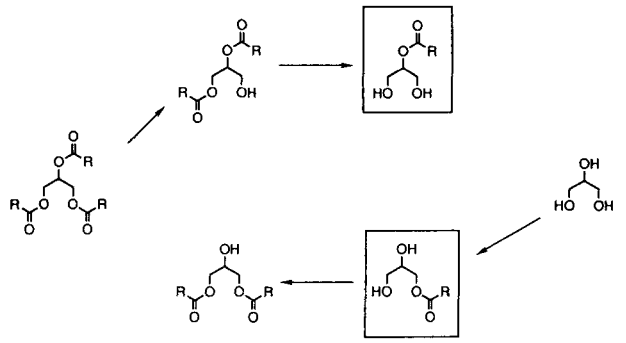


FIG. 2. Esterification of glycerol with 1,3-specific lipases (acyl transfer from triacylglycerols).

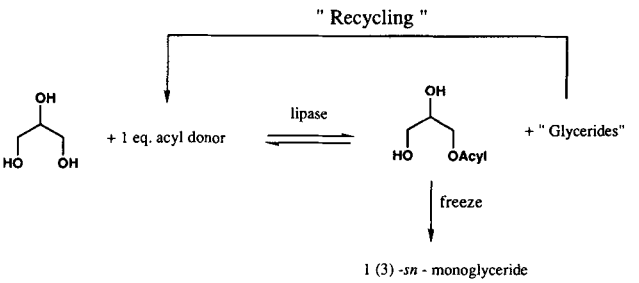
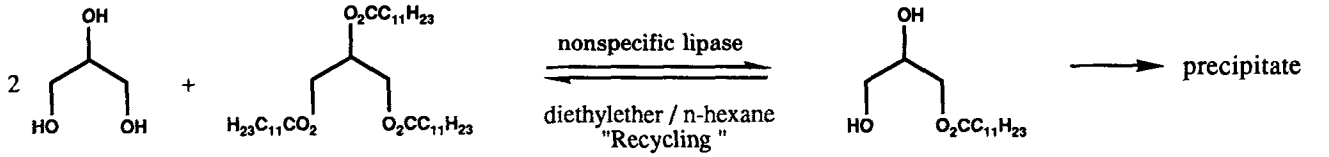


FIG. 3. Synthesis of regioisomerically pure 1(3)-monoacylglycerols; isolation and recycling of by-products.

purification steps. To avoid this, a simple method for the continuous separation of the desired products from the reaction mixture had to be developed. Furthermore, to achieve quantitative conversion of the acyl donors into the desired products, an effective recycling method of all undesirable materials (including 2-monoacylglycerols, diacylglycerols, triacylglycerols) had to be incorporated.

Both goals were achieved simply by a compartmental separation of the two steps of the process—synthesis and isolation (Fig. 3). The enzymatic esterification is carried out in the reactor vessel with stoichiometric quantities of glycerol and the corresponding acyl donor under the desired reaction conditions. The solution of the obtained reaction mixture is circulated into a second vessel—termed separator—in which the desired monoacylglycerols are separated at lower temperatures.

Crucial for success of this method is the choice of the solvent or solvent mixture in which the desired, more polar 1(3)-monoacylglycerols are less soluble than all of the other products, at least at the low temperature employed. While the desired products are precipitating out in the cooled separator, all other products, as well as unreacted acyl



[4]

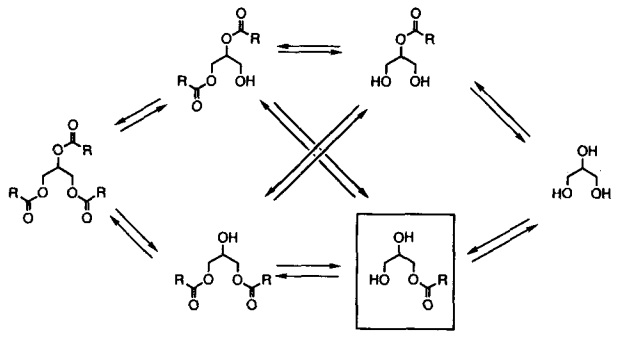


FIG. 4. Esterification of glycerol with unspecific lipases. Acyl transfer from triacylglycerols.

donors, remain in solution and are fed back to the reactor, which contains both the enzyme and the glycerol on the solid support. Interestingly enough, unspecific lipases can be successfully employed to achieve a complete conversion of all acyl groups into the desired products. This is particularly important when natural fats and oils are used as acyl donors. As is obvious from Eq. [4], all acyl groups of natural glycerides should be used, in particular those in the 2-position. This is further illustrated in Figure 4.

It is interesting to note that for conversion of triacylglycerols and natural fats and oils into regioisomerically pure 1(3)-monoacylglycerols, unspecific lipases are best suited. The success of the method was demonstrated by using numerous acyl donors, such as free fatty acids, fatty acid methyl esters, vinyl esters, synthetic triacylglycerols and natural fats and oils. Optimization of the solvent mixture and the choice of the optimal temperature for the separation process allowed a preparation of various solid monoacylglycerols in high yields and isomerical purity without the need for further purification steps (Table 2). The products are completely colorless and odorless. From a regulatory point of view, it is important that these monoacylglycerols obtained from natural fats and oils are truly "natural" in every respect as defined by legislature. Approval of government bodies, such as the FDA, for the use of these material in pharmaceutical, cosmetic and food applications should therefore pose no problem.

The described procedures for the synthesis of regioisomerically pure 1(3)-monoacylglycerols provide ready access to these interesting compounds on a synthetically useful scale. Good yields were achieved in the transformations, and products of high chemical and isomeric purity are obtained without any additional purification steps. The convenient access to these materials in synthetically useful quantities should greatly stimulate practical applications in pharmaceutical and cosmetic areas of research. Their potential for synthetic applications can now be studied in detail. Studies of this kind are currently in progress at our laboratory.

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